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The Conversion of Orotic Acid to Uridine Nucleotides *in vitro*

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The conversion of radioactive orotic acid to 5′-uridine monophosphate and three 5′-uridine pyrophosphate nucleotides was effected by dialyzed particle-free extracts from a number of animal and avian tissues. Ribose-5-phosphate, ATP and magnesium ion were required for the conversion. The conversion could also be obtained in extracts metabolizing hexose diphosphate. Chromatographic evidence indicated the 5′-uridine nucleotides to include uridine diphosphate and uridine triphosphate. Uridine and uracil were found not to be intermediates in the conversion although these compounds could be produced from the uridine nucleotides by enzymes present in the extracts.

By the use of a partially purified enzyme preparation from chicken liver it was demonstrated that ribose-5′-phosphate and ATP react to produce ribose triphosphate, presumably 5-phosphoribosyl pyrophosphate, and that this compound is the source of the ribose phosphate moiety of the uridine nucleotides. Ribose-1,5-diphosphate was inactive with this enzyme preparation, even in the presence of ATP. The 5′-uridine monophosphate was the first of the uridine nucleotides to be formed; the other nucleotides arose by the action of phosphorylating enzymes present in other enzyme fractions.

Recent evidence has demonstrated that orotic acid is a key intermediate in a series of metabolic reactions whereby the uracil of the polynucleotides is formed. Orotic acid was first found to be utilized as a specific precursor of the pyrimidines in the nucleic acids of the rat by Arvidson *et al.*[1] (*cf.* also [2]). It has since been shown to be a normal intermediate in the formation of the polynucleotides [3] and the individual steps in its enzymatic synthesis from aspartic acid, ammonia and carbon dioxide have been described [4–7]. In the liver of the living rat the compound was found to be converted almost quantitatively into the free uridine nucleotides ** which were apparently closely involved as pre-

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** 5′-Uridine phosphate (UMP), 5′-uridine diphosphate (UDP), 5′-uridine triphosphate (UTP) and several 5′-uridine pyrophosphoglycosyl compounds. Other abbreviations used in this paper are: 5′-adenosine nucleotides (AMP, ADP and ATP) and diphosphopyridine nucleotide (DPN).

cursors of the polynucleotide uracil [8]. The widespread occurrence of many enzymatic reactions of the uridine nucleotides has been the subject of numerous other reports (*cf.* Ref.[9]). Further detailed study of the reaction sequences involving orotic acid is of great importance in learning the overall mechanism of the formation of the polynucleotides.

In a continuation of the investigations previously carried out with living rats and rat liver slices, we have been engaged in studying the enzymatic processes by which the orotic acid is utilized. The object of this paper is to describe studies on the formation *in vitro* of the uridine nucleotides and to attempt to correlate the *in vivo* and *in vitro* processes. During the course of this project * the publications of Kornberg *et al.*[11,12] have appeared, which elucidate the enzymatic steps whereby orotic acid reacts with 5-phosphoribosyl pyrophosphate to form orotic acid ribotide and the subsequent decarboxylation of this product to form UMP. Our work has been done with different sources and procedures and is found to confirm and supplement that of Kornberg *et al.* A preliminary publication has appeared [13].

EXPERIMENTAL

Orotic acid-2-^{14}C containing about 1 μC per μmole was prepared by oxidation of $K^{14}CN$ to KCNO, by the method of Gall and Lohman [14] and the condensation of the KCNO with aspartic acid, by the method of Nyc and Mitchell [15].

Preparation of the particle-free extracts. The tissue was homogenized [10] in 4 volumes of 0.13 *M* KCl – 0.01 *M* $MgCl_2$ – 0.015 *M* potassium phosphate, pH 7.2, in a Potter-Elvehjem homogenizer and centrifuged at 60 000 – 70 000 g for 30 minutes in a Spinco centrifuge. Fat particles were partially removed by decantation of the supernatant solution through cotton or glass wool. The extracts were dialyzed against the same medium (50 ml of extract *versus* two successive 2 liter volumes) for 12 – 15 hours. All these operations were at 0 – 5° **.

Incubation. In the "hexose diphosphate system" the amounts of substrate used were: 0.5 μmole of orotic acid-2-^{14}C, 20 μmole of hexose diphosphate, 1.5 μmole of DPN, 120 μmole of nicotinamide and 2.0 ml of the tissue extract. In the "ribose phosphate system" the amounts used were: 0.5 μmole of orotic acid-2-^{14}C, 20 μmole of ribose phosphate, 20 μmole of 3-phosphoglyceric acid, 5 μmole of ATP and 2.0 ml of the tissue extract. In some experiments 3 mg of a muscle enzyme preparation [16] were also added to ensure utilization of the phosphoglyceric acid for regeneration of the ATP. The total volumes of both incubation systems were 4.0 ml. The incubations were carried out in 25 ml. Erlenmeyer flasks, which were shaken at 37° for 40 minutes.

At the end of the incubation period, the flasks were chilled and made to 0.4 *N* perchloric acid. The precipitated proteins were centrifuged and washed once with 1 ml of cold 0.2 *N* perchloric acid. The perchloric acid was neutralized in an ice bath with KOH, using phenol red as an internal indicator. The neutralized extract was decanted onto the chromatographic column through glass wool to remove the $KClO_4$ precipitate, which was washed once with cold water. The $KClO_4$ retained less than 2 % of the ^{14}C.

Chromatography. The principle of the gradient elution procedure previously described [17] was used although the technique was simplified to permit rapid routine work. Four columns, 6 cm × 1 cm, of Dowex-2 (formate) were eluted simultaneously by increasing concentrations of eluent from one 500 ml mixing flask, which received the

* The original observation of Saffran and Scarano [10], that 5-phosphoribose is utilized by pigeon liver extracts to convert adenine to AMP, provided a valuable stimulus for the work with orotic acid.

** The activity of the extract in the "hexose diphosphate system" diminished 20—40% during the dialysis. The larger losses were found at the higher temperatures and longer times of dialysis.

concentrated eluent by gravity flow from the reservoir flask. Fractions of 5 ml volume were collected by an automatic collector. The mixing flask initially contained water and the reservoir flask 6 *N* formic acid; after 25 fractions had been collected from each column (resulting in a concentration of formic acid in the mixing flask of about 4 *N*) the reservoir flask was changed to contain 4 *N* formic acid — 1.5 *M* ammonium formate. Another 25 fractions were collected so that the final concentration of eluent in the mixing flask was about 4 *N* formic acid — 1 *M* ammonium formate. All of the ^{14}C was eluted from the columns in this way.

The light absorption of the fractions was read at 260 mμ in a Beckman spectrophotometer, Model DU, primarily to detect the adenosine nucleotide peaks. Small aliquots of each fraction were plated on aluminum planchets, dried at 60—80° to remove ammonium formate, and the radioactivity was counted 30 seconds in a Tracerlab Sc-18 windowless flow counter. The procedure could readily detect the presence in one fraction of 0.5 % of the ^{14}C used in each experiment. The fractions containing each radioactive peak were later combined and the amounts of ^{14}C were determined again by plating and counting more accurately. The amount of ^{14}C in each peak recorded in Tables 1—4 is given as the percent of the total *summated* radioactivity in the entire chromatogram. The reproducibility of recovery of the radioactivity used in the experiment was within about ± 10 %.

The location of the various peaks found on these chromatograms was as follows: Uridine and uracil ("U") were in fraction 1 (containing the effluent from the sample placed onto the column) and fraction 2, AMP was in fractions 6—7, UMP in 18—20, orotic acid and ADP in 22—24, "UX" in 32—33, "UDP" and ATP in 35—37 and "UTP" in 42—43. When the columns were identical with regard to dimensions and flow rate the position of these peaks was reproducible. The "UX", "UDP" and "UTP" peaks are so designated because their chromatographic behaviour corresponded to that of the known compounds described in previous work [17], although in the present work they were not rigorously identified.

Enzyme fractionation. The fractionation procedure was used for rat, pigeon and chicken liver preparations. It will be described here for chicken liver.

The particle-free extract from 60—80 g of liver was fractionated by the addition of solid ammonium sulfate to obtain the protein precipitable between 35 % (0.245 g of ammonium sulfate per ml) and 45 % of saturation. This fraction contained most of the activity towards orotic acid. The precipitate was dissolved in 30 ml of 0.03 *M* phosphate buffer, pH 6.6, and dialyzed against 2 successive 2 liter volumes of the same buffer, at 1—2°, for a total of 2 hours. The protein solution was made to 50 ml with the buffer and 10 ml of methanol was added at the rate of 1 ml per minute while the temperature was being lowered to —5°. After another 10 minutes the precipitate was centrifuged at —5°. This precipitate contained little activity. A mixture of 5 ml of 0.08 *M* sodium acetate buffer, pH 4, and 1 ml of methanol was added slowly at —5°, followed by centrifugation as before. This precipitate contained most of the activity. The pH of the supernatant solution was 6.0. A third precipitate obtained by lowering the pH to 5.5 in a similar way was usually inactive. The active fraction, termed the "pH 6 methanol" fraction, was dissolved in 10 ml of 0.02 *M* phosphate buffer, pH 7.2, lyophilized and stored at 0°. This preparation retained its activity well for at least 2 weeks.

Direct spectrophotometric assay of enzyme activity. With the partially purified enzyme fractions a direct spectrophotometric assay for the disappearance of orotic acid was possible. A solution of 0.3 ml volume containing 0.1 μmole of non-radioactive orotic acid, 0.2 μmole of ATP, 0.2 μmole of ribose-5-phosphate, 1.25 μmole of $MgCl_2$, 15 μmole of phosphate buffer, pH 7.4, and 0.5—1.5 mg of lyophilized enzyme was incubated for periods up to 2 hours at 37°, then chilled during the addition of 1.25 ml of cold 0.5 *N* perchloric acid, and centrifuged. For each determination a second solution was prepared and treated identically with the first except that the orotic acid was omitted. The supernatant solutions, in 1 ml quartz cuvettes, were read at 260, 280, 300 and 350 mμ against an appropriate perchloric acid blank. To obtain the change in spectrum resulting from the action of the enzymes on the orotic acid, the readings of the "minus orotic" sample were subtracted from those of the "orotic" sample. This correction was necessary because during the incubation changes in the spectrum occurred, apparently arising from the protein. As a measure of the initial concentration of orotic acid, a solution of orotic acid in perchloric acid (without substrates or enzyme) was also prepared and analyzed in

an identical way. The amount of orotic acid converted was calculated directly from the decrease in the corrected reading at 300 mμ, where neither ATP nor UMP absorb. The readings at 260 and 280 were made to check on the formation of UMP, which absorbs maximally at 260 mμ. Small corrections for the light absorption of traces of colloidal protein were made by substracting the readings made at 350 mμ from the readings at 300 mμ.

Preparation of ribose triphosphate. A solution of 25 ml volume, containing 150 μmole of ribose-5-phosphate, 100 μmole of ATP, 100 μmole of $MgCl_2$, 950 μmole of phosphate buffer, pH 7.4, 900 μmole of NaF, and 200 μmole of reduced glutathione, was incubated at 37° with 20 mg of the "pH 6 methanol" enzyme. These conditions are basically those of Kornberg, *et al.*[11]. The reaction was followed by the use of adenylic acid deaminase to determine the amount of AMP produced from the ATP [18]. For this determination, 15 μl aliquots were removed into 1.0 ml of pH 6.0 citrate buffer and the change in light absorption at 265 mμ was determined upon the addition of the deaminase. When no further formation of AMP could be detected by this test (about 1.5 hours), the reaction was stopped by pouring the solution directly onto a 12 cm × 2 cm Dowex-1 (formate) column. The column was washed well with water and gradient elution was begun with water in the 500 ml mixing flask and with 2 *M* sodium formate of pH 5 in the reservoir flask. About 100 fractions of 6 ml volume were collected; these were analyzed for their ribose content and light absorption at 260 mμ. The ribose triphosphate was eluted in 5 fractions between the AMP and ADP. These fractions were low in light absorption. The combined fractions were adjusted to pH 7.0 with KOH, 0.5 ml of 2 *M* barium acetate was added, and the ribose triphosphate was precipitated by 1 volume of cold ethanol. After one hour at 0°, the precipitate was collected by centrifugation and washed successively with cold 50 % ethanol, ethanol and ether. Analysis of the best of these preparations, used for the work described later, indicated a ratio of ribose: acid-labile phosphate: total phosphate of 1 : 2.04 : 3.01 and a purity of 42 % (calculated with 2.5 Ba per ribose).

Ribose-1,5-diphosphate was prepared from ribose-1-phosphate and glucose diphosphate with crystalline mutase according to Klenow [19]. Purification was effected by gradient elution chromatography with 1 *M* sodium formate, pH 5, and precipitation with barium acetate as described for ribose triphosphate. The substance had a ratio of ribose: acid-labile phosphate: total phosphate of 1.0 : 0.99 : 2.06 and was 77 % pure, calculated as the dibarium salt.

Uridine-2-^{14}C and uracil-2-^{14}C were prepared from radioactive UMP by hydrolysis with 98 % formic acid at 175° for 4 hours followed by chromatography on a starch column [20].

RESULTS

The conversion of orotic acid to the uridine phosphates was initially tested in homogenates of rat liver metabolizing hexose diphosphate. The chromatographic analysis for the orotic acid, uridine nucleotides and adenosine nucleotides was employed. Small conversions of the orotic acid to uridine nucleotides and the "U" peak were noted when the homogenates were metabolizing the hexose diphosphate either aerobically or anaerobically. In both systems the amount of conversion appeared to be dependent upon the length of time the ATP levels were maintained. Both the maintenance of ATP and the conversion of orotic acid were then found to be greatly improved in the glycolyzing system when the particulate cell components were first removed by high speed centrifugation. Anaerobic incubation was not necessary in the particle-free system. The enzymes metabolizing orotic acid to the uridine nucleotides occur therefore in the non-particulate portion of the cytoplasm and, judging by the recovery of total activity, appear to be located primarily in this fraction.

A large number of exploratory experiments were carried out by incubating extracts of rat and pigeon liver with various combinations of substrates, activators and inhibitors. The relative amounts of orotic acid converted to

"U" and the various uridine nucleotides as well as the relative amounts of the adenosine nucleotides were determined chromatographically. When the particle-free extracts were dialyzed they were no longer active in the conversion unless hexose diphosphate, DPN and magnesium ion were added. (The addition of ATP was not necessary; however traces of ATP, perhaps arising from AMP in the DPN, were detectable chromatographically at the end of the incubations.) Phosphoglyceric acid was capable of partially replacing hexose diphosphate in this system. No conversion was obtained in the absence of either magnesium ion or DPN, nor in the presence of the glycolysis inhibitor iodoacetate *. It was apparent that the hexose diphosphate was serving both as a source of energy (*via* glycolysis and ATP) and of the ribose moiety of the uridine nucleotides.

The dialyzed extracts of pigeon liver (but not of rat liver *) could also be activated by addition of ribose-5-phosphate, provided ATP and magnesium ion were also present. DPN was not required. The full activity of the extract was not obtained in this way, apparently due to the destruction of ATP, since no ATP was detectable at the end of the incubation. However, the ATP level could be maintained and full activity obtained in both rat and pigeon liver extracts by the addition of phosphoglyceric acid, to serve as an energy source for the regeneration of ATP. (Phosphoglyceric acid in the absence of DPN could not replace ribose phosphate in this system.) From these data and from the work of Saffran and Scarano [10], it seemed probable that the ribose phosphate was providing the ribose moiety of the uridine nucleotides much more directly than did the hexose diphosphate and that ATP was a necessary reactant. Knowledge of the multiplicity of enzymes in the extract could not permit such an assumption to be conclusive, however **.

In the chromatograms of these reaction mixtures, five radioactive peaks in addition to the orotic acid were usually obtained. The first of these, the "U" peak, was in some experiments found to contain primarily uracil and some uridine as the radioactive components. For this work a warm butanol extract of the dried "U" peak (collected from a number of experiments) was chromatographed on a starch column with butanol-water [20]. The radioactive peaks were identified by their spectrum, R values and by recrystallization with authentic carrier uracil (solvent: water) and uridine (solvent: alcohol-ethylacetate).

The second peak contained UMP, which was identified by spectrum, phosphorus and ribose content, and by liberation of the phosphate group by snake venom 5′-nucleotidase [21, 22]. The three following peaks, labeled "UX", "UDP" and "UTP", corresponded in their chromatographic behaviour to the UDP-hexose, UDP and UTP of rat liver [17, 22], and contained uridine-5′-phosphate which could be liberated by hydrolysis with 1 N H_2SO_4 at 100° for 15 minutes. From larger scale incubation mixtures, the "UX" and "UDP" peaks were isolated by rechromatography with pH 5 ammonium formate [17].

* In pigeon liver extracts, which destroyed ATP much less rapidly than did rat liver extracts, some conversion of orotic acid could be obtained in the presence of iodoacetate by the addition of sufficient ATP (10—20 μmoles).

** Ribose phosphate and magnesium ion *in the presence of DPN* were also capable of generating ATP and effecting the conversion of orotic acid.

Analysis of both peaks indicated the ratio of uridine: total phosphate to be 1 : 2. The presence of either a hexose or a pentose in the "UX" peak could not be clearly determined*. Insufficient "UTP" was obtained for clear analyses. All of these peaks were usually found in the chromatograms of both the "hexose diphosphate" and "ribose phosphate" systems. The relative amounts of the peaks varied with the length of incubation, ATP level, age of the extract and the tissue source.

A number of tissues and one micro-organism were examined for their ability to carry out these conversions in the "ribose phosphate system". In these incubations, 3 mg of a muscle enzyme preparation [16] was added to assist maintenance of ATP; the muscle preparation itself had no activity towards orotic acid in the "ribose phosphate system". The results are presented in Table 1.

Table 1. Formation of uridine nucleotides from orotic acid-2-^{14}C by particle-free extracts of various tissues.

Tissue	Percent of total ^{14}C in chromatographic peaks					
	"U"	UMP	OR	"UX"	"UDP"	"UTP"
Pigeon Liver *	1	19	36	9	22	13
Chicken Liver	2	18	0	14	25	41
Rat Liver	11	52	0	27	9	1
Mouse Liver	24	53	0	20	3	0
Chicken Pancreas	0	0	0	4	2	94
Rat Kidney	19	7	68	5	1	0
Rat Spleen	41	47	0	6	6	0
Rat Heart	0	0	98	0	2	0
Mouse Kidney	14	27	53	5	1	0
Mouse Tumor **	4	23	25	16	15	17
E. coli ***	16	9	30	8	14	23

Each extract was prepared from 400 mg of tissue and incubated for 40 minutes at 37° with 0.5 μmole of orotic acid (500 000 c/min) in the "ribose phosphate system" (see text) with the addition of phosphoglyceric acid and muscle enzyme [16]. Cold perchloric acid extracts of the reaction mixture were made and chromatographed on Dowex-2 (formate) by elution with formic acid and ammonium formate. The radioactive peaks obtained are labeled "U" for uracil and uridine, UMP for uridine monophosphate, OR for remaining orotic acid, and "UX", "UDP" and "UTP" for the uridine pyrophosphate nucleotides.

* Phosphoglyceric acid and muscle enzyme *not* added.

** Spontaneous mammary tumor from C3H mice, kindly provided by Dr. Georg Klein.

*** Cell-free extract (prepared by repeated freezing and thawing from 50 mg of lyophilized *Escherichia coli*).

* The "UX" peak of an experiment with rat liver extract, ribose phosphate and phosphoglyceric acid was later rechromatographed and found to contain 43 % UDP-Glucose and 42 % UMP (the latter arising by degradation). For the spectrophotometric demonstration of the UDPG (by means of uridyl transferase, phosphoglucomutase and Zwischenferment [18]), we wish to thank Civ. Ing. Agnete Munch-Petersen.

A number of experiments were carried out with rat and pigeon liver in attempts to determine which of the radioactive products was formed first. Time curves indicated UMP to be the dominant peak at early time intervals (*cf.* Ref.[13], Fig. 1). The addition of non-labeled UMP to the incubation mixture (Table 2) increased the relative amount of incorporation into the UMP peak initially and greatly diminished the incorporation into the uridine pyrophosphates. This indicated the UMP to be the first uridine nucleotide product. The large shift of radioactivity into the "U" peak in the later time intervals of the experiment was interpreted to mean that the "U" peak was formed primarily by degradation of the UMP*. The addition of non-radioactive uridine did not greatly affect the distribution of radioactivity, which eliminated this compound from consideration as an intermediate in the conversion. Later work with partially purified enzymes verified these interpretations.

Table 2. Formation of uridine nucleotides from orotic acid-2-^{14}C: Effect of added pools of non-radioactive UMP or uridine.

Non-radioactive pool	Incubation Time Minutes	Percent of total ^{14}C in chromatographic peaks					
		"U"	UMP	OR	"UX"	"UDP"	"UTP"
UMP	5	0.3	2.4	96.0		0.8	0.5
UMP	10	1.7	5.7	89.0		1.9	1.7
UMP	40	30.5	10.4	55.4	1.0	2.7	
Uridine	40	1.7	0.4	63.0	12.7	9.5	12.7
None	40		1.0	59.0		40.0	

Particle-free extracts from 200 mg of rat liver were incubated at 37° with 0.5 μmole of orotic acid (500 000 c/min) in the "hexose diphosphate system" (see text). Pools of non-radioactive UMP (4 μmole) or uridine (2 μmole) were added as noted. The reaction mixtures were analyzed as in Table 1.

The enzymes of the particle-free extract were fractionated in order to determine which substrates and products were immediately concerned in the utilization of orotic acid. The fractionations were carried out with preparations from rat, pigeon and chicken livers. The latter proved to be the best source for the larger scale preparations, although similar results were obtained with all these tissues. The preparation of the active 35—45 % ammonium sulfate and the "pH 6 methanol" fractions is described earlier.

The 35—45 % ammonium sulfate fraction was tested in the "ribose phosphate system" (with the addition of the muscle enzyme preparation) and analyzed by chromatography. The results (Table 3) indicated that the enzymes

* That the "U" peak may be further metabolized is indicated by Fig. 1 [13], in which the "U" peak was formed initially and disappeared at later time intervals. This may represent conversion back to the uridine nucleotides or possibly complete degradation [24]. In other experiments with rat liver extracts plus phosphoglyceric acid and muscle enzyme, some formation of uridine nucleotides from uridine has been noted (unpublished).

degrading UMP to "U" were nearly eliminated and that the enzymes phosphorylating the UMP were diminished. Other experiments showed that the destruction of ATP by this preparation was not rapid.

Table 3. Formation of uridine nucleotides from orotic acid-2-^{14}C by 35—45 % Am_2SO_4 enzyme fraction from rat liver.

Incubation Time Minutes	Percent of total ^{14}C in chromatographic peaks					
	"U"	UMP	OR	"UX"	"UDP"	"UTP"
5		1.5	95.0	1.0	2.0	0.5
10		5.5	85.5	5.5	2.5	1.0
20	1.0	12.5	67.0	9.0	7.5	3.0
40	5.0	41.0	30.0	12.0	11.0	1.0

An amount of the ammonium sulfate fractionated enzyme containing 23 mg of protein was incubated in the "ribose phosphate system" with phosphoglyceric acid and muscle enzyme [16] (see text).

The "pH 6 methanol" enzyme preparation was tested with ribose phosphate, ATP and magnesium ion by both the chromatographic and spectrophotometric analyses. When relatively small amounts of ATP and large amounts of ribose phosphate were employed (Table 4) only UMP was formed from the orotic acid, indicating that the enzymes causing the degradation of the UMP were absent and that ATP-ase activity was negligible. Although the inclusion of phosphoglyceric acid and the muscle enzyme in the incubation mixture caused the formation of small amounts of the uridine pyrophosphates, the results indicated that the enzymes phosphorylating the UMP had been greatly reduced by the fractionation and were not essential for the reaction. Table 4 also shows that when either radioactive uridine or uracil replaced orotic acid in the incubation mixture there was no formation of uridine nucleotides. The UMP was thus demonstrated to be the first of the uridine phosphate compounds to be formed.

It appeared likely from the work of Kornberg *et al.*[11,12] that this enzyme preparation contained at least three enzymes concerned with the orotic acid utilization. In order to test for the reactions described by Kornberg *et al.*, ribose triphosphate was prepared using the "pH 6 methanol" fraction. The isolated ribose triphosphate (presumably 5-phosphoribosyl pyrophosphate) was found to be capable of replacing ribose monophosphate and ATP, when tested with the "pH 6 methanol" fraction in the direct spectrophotometric assay (Table 5). No activity was observed with the ribose triphosphate in the absence of magnesium ion. A sample of authentic ribose diphosphate, prepared according to Klenow [19] was found to be inactive with this preparation, even in the presence of ATP. Since no accumulation of orotic acid ribotide could be detected with any of the extracts or enzyme preparations employed it was not possible to make further tests for the mechanism described by Kornberg *et al.*

Table 4. Formation of uridine nucleotides from orotic acid-2-^{14}C by "pH 6 methanol" enzyme fraction. Test of activity with uracil and uridine.

Experiment	Radioactive Substrate	Time, Minutes	Percent of total ^{14}C in chromatographic peaks					
			"U"	UMP	OR	"UX"	"UDP"	'UTP"
I	Orotic acid	5		2	98			
I	»	10		6	94			
I	»	20		22	78			
I	»	40		32	68			
I	»	60		42	58			
I	Uridine	20	100					
I	Uracil	20	100					
II	Orotic acid	30		40	50		2	8

Experiment I: The incubation mixtures contained 5 μmole of ribose-5-phosphate, 0.6 μmole of ATP, 20 μmole of $MgCl_2$, 60 μmole of phosphate buffer, pH 7.4 and 8 mg of lyophilized enzyme (6.8 mg of protein). The radioactive substrates were 0.5 μmole of orotic acid (500 000 c/min) or 0.05 μmole of uridine (37 500 c/min) or 1 μmole of uracil (750 000 c/min). Total volumes were 2.5 ml.

Experiment II: The incubation mixture contained 3 μmole of ribose-5-phosphate, 3 μmole of ATP, 20 μmole of $MgCl_2$, 60 μmole of phosphate buffer, pH 7.4, 0.5 μmole of orotic acid (500 000 c/min) and 15 mg of lyophilized enzyme (10.5 mg of protein) in a total volume of 3.0 ml.

Different enzyme preparations were used in Experiments I and II. The incubation temperature was 37°.

Table 5. Conversion of orotic acid by "pH 6 methanol" enzyme fraction. Test of ribose phosphate, ribose diphosphate and ribose triphosphate in direct spectrophotometric assay.

Ribose compound	μmoles of orotic acid converted at given incubation times			
	15 min.	30 min.	45 min.	60 min.
RMP				0.000
RMP + ATP	0.010	0.021	0.032	0.039
RDP				0.000
RDP + ATP				0.000
RTP	0.012	0.023	0.028	0.031
RTP + ATP				0.036

1.0 mg of lyophilized enzyme (containing 0.8 mg of protein) was used for each determination in the direct spectrophotometric assay (see text). The initial amounts of the reactants in the assay were: 0.10 μmole of non-radiactive orotic acid, 0.20 μmole of ribose-5-phosphate (RMP), 0.15 μmole of ribose-1,5-diphosphate (RDP), 0.12 μmole of ribose triphosphate (RTP), and 0.20 μmole of ATP.

DISCUSSION

The objective of this work has been to examine some of the enzymatic steps in the utilization of orotic acid for synthesis of the polynucleotides in living tissues. It has been established that the initial reaction in the utilization is the condensation of orotic acid and ribose triphosphate with the subsequent formation of UMP. The enzymes of the non-particulate portion of the cytoplasm were responsible for these reactions. (It was, however, not determined whether such enzymes are *absent* in the cell particles.) Other enzymes of this cytoplasmic fraction were found to be capable of splitting the UMP to produce uracil and uridine, as well as of phosphorylating it to form several uridine pyrophosphate nucleotides. A different cell fraction, the mitochondrial fraction of the cytoplasm, appears to be responsible for the reactions leading to the *formation* of orotic acid [5].

Kornberg *et al.*[11, 12], have described the intermediate steps in the conversion of orotic acid to UMP. An enzyme prepared from pigeon liver reversibly catalyzed the reaction of ATP with ribose-5-phosphate to form 5-phosphoribosyl pyrophosphate. Enzymes from both pigeon liver and yeast caused coupling of the latter compound with orotic acid by elimination of pyrophosphate, followed by decarboxylation of the product to form UMP. The reversible formation of orotic acid ribotide as an intermediate was demonstrated with a yeast preparation freed of orotic acid ribotide decarboxylase activity. The decarboxylation step appeared to be essentially irreversible. We have confirmed the initial coupling of orotic acid with ribose triphosphate and, although our enzyme preparation was not free of decarboxylase activity so that the formation of orotic acid ribotide was not demonstrated, it seems probable that our system followed the reactions described by Kornberg *et al.*

A number of tissues have been examined for their ability to convert orotic acid to uridine nucleotides. Of these, liver, pancreas and spleen were relatively active, tumor and kidney were less active and heart muscle was nearly inactive. The amount of activity toward orotic acid appears to correlate roughly with the content and "turnover" of the ribonucleic acid in the tissues examined. The differences in the relative amounts of the uracil compounds formed by these tissues probably reflects different balances among the various enzyme activities involved. Such differences may be of value in preparative work; the high yield of "UTP" with the chicken pancreas is especially noteworthy in this respect. In previous work [2] a rapidly growing Flexner-Jobling tumor and other extrahepatic tissues of the *living* rat utilized only small amounts of the injected orotic acid for nucleic acid synthesis, in apparent discrepancy with work on other precursors in *in vitro* systems. The present report supports the suggestion that many tissues (including tumors) have the potential ability to utilize orotic acid, but that factors such as circulation and absorption greatly affected the results obtained *in vivo*. The conversion of orotic acid to uridine nucleotides has also been found to occur in *E. coli* (present report), yeast [11, 12], *S. aureus* [23] and mouse ascites tumor *.

* The conversion of orotic acid-4-^{14}C to the free uridine and cytidine nucleotides has also been found in ascites tumor by Dr. Hanns Schmitz (personal communication) and in oxidizing fortified homogenates of rat liver by Liselotte I. Hecht (presented at the Meeting of the American Association for Cancer Research in April, 1954).

With regard to the activity of the enzyme system converting orotic acid to UMP, the extract from 1 gram of liver could convert 1.25 μmole of orotic acid in 30—40 minutes. Although this activity does not appear great, it may be calculated that the total uridine nucleotides of the liver (about 1 μmole per gram of fresh weight) could thus be renewed from orotic acid in about one-half hour. This rate does not seem to be grossly inadequate for the supply of the uracil moiety entering the nucleic acids. (The total ribonucleic acid contains roughly 3 μmole of uracil per gram fresh weight of liver and the renewal time of the polynucleotides is of the order of many hours [2]). Whether the rate of *formation* of orotic acid in the liver is adequate to account for the uridine nucleotides formed remains to be determined. Reichard [5] has shown the rate of synthesis of ureidosuccinic acid, a precursor of orotic acid, to be more than adequate. Other pathways of uridine nucleotide formation may well exist; in fact work with other organisms indicates that uracil and possibly uridine may also be utilized to varying extents [23-26] for synthesis of polynucleotides. Whether or not this utilization proceeds through the uridine nucleotides has not been established.

The cytosine of the polynucleotides becomes labeled when orotic acid is used as precursor in living rat [1,2]. Under these conditions the free cytidine nucleotides of the liver also become labeled to a specific activity one-fourth to one-half that of the uridine nucleotides *. In the present work with the particle-free extracts, no significant formation of radioactive cytidine phosphate was noted. This negative finding may indicate either that the system used was inadequate with regard to substrates or that some of the cell particulates are involved. Further investigation of the formation of cytidine phosphate is in progress.

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